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Nucleic acid-based drugs as antiviral agents

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Abstract

Nucleic acid-based drugs are promising classes of therapeutic agents that have important clinical applications for the prevention and treatment of viral diseases. This review highlights the applications of three main classes of oligonucleotides that are designed to suppress, inhibit and/or knock out virus gene expression. Antisense oligonucleotides have a high level of specificity and affinity for target genes and have been shown to be efficacious in the treatment of various virus infections, including HIV, hepatitis, and human papilloma viruses. Catalytic DNAzymes are DNA-based enzymes with considerable potential as they have the ability to cleave RNA transcripts at purine-pyrimidine junctions, and they do so with high level of target site flexibility. Similarly, ribozymes are catalytic RNA enzymes engineered to cleave target RNA in a sequence-specific manner. Among these are the hammerhead and hairpin ribozymes that are particularly useful for gene therapy of clinically relevant virus diseases. This review will focus on the design, mode of action, efficacy of these groups of oligonucleotides, and the evaluation of these agents from *in vitro* activity to efficacy in animal protection studies, through to

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safety in clinical trials.

Introduction

Conventional chemotherapy has required that drugs against viral targets are either discovered by serendipity or are screened from chemical libraries of tens or hundreds of thousands of compounds. This is a slow, expensive process and even until recently has produced relatively few therapeutic antiviral drugs. The advent of biotechnology and molecular genomics has meant that the information from the sequence of the relatively small viral genomes can be exploited to target viruses in a highly specific manner through gene ablation or gene expression. In gene ablation approaches, oligonucleotides can be employed which bind in a highly specific manner by Watson-Crick base pairing to the viral genome, transcripts and/or regulatory sites. By various steric blockade or catalytic means, viral replication can be inhibited by targeting conserved genes. Using gene expression approaches, genes that encode for protective viral antigens can be cloned into plasmid DNA that contains the regulatory sites. Genetic vaccination can then be achieved using a plasmid construct that results in the induction of long-lasting cellular and humoral immune responses in the host. In this manner the host's own protein synthesis machinery and immune system can be employed to confer antiviral protection, without exposure to the potential hazards of a live or attenuated vaccine. Alternatively, non-specific antiviral resistance can also be induced in the host using ds RNA or CpG oligonucleotides.

In this review we outline several antiviral approaches that utilize nucleic acid-based therapeutic agents for the experimental therapy of antiviral diseases. Nucleic acids such as antisense oligonucleotides, ribozymes and DNAzymes have been designed and evaluated for their antiviral activities in tissue culture systems and in animal protection studies. These examples provide evidence that nucleic acid-based drugs have the potential to be used as an economical, expedient and powerful approach to antiviral therapy.

A. Oligonucleotide agents

Antiviral Antisense

Over two decades ago Zamecnik and Stephenson [1,2] demonstrated that the RNA translation, virus replication and cell transformation of Rous sarcoma virus could be inhibited by a 13-mer deoxyribonucleotide that was complementary to the RSV repeat terminal sequences. This provided the first evidence that an antisense oligodeoxynucleotide (ODN) could act as an antiviral agent. Studies of antisense ODN over the past several years have demonstrated that the mechanisms of action are complex and may include steric hinderance of ribosome and reverse transcriptase action, rendering the target RNA susceptible to RNAse H cleavage, blocking viral binding and enhancing immunostimulation via CpG motifs [3-6]. The susceptibility of phosphodiester ODN to *in vivo* nuclease activity resulted in the development of a range of nuclease-resistant chemical modifications either to the phosphate backbone (ie. phosphorothioate, phosphorodithioate, methyl phosphonate), to the 2'-position on the ribose sugar moiety (2'-*O*-methyl, 2'-methoxy ethyl) or to the oligonucleotide structure (3'-inversion) [5]. Suitably modified antisense ODNs are 'designed' for antiviral activity by targeting hybridization to an essential sequence such as the AUG start codon or by screening a range of antisense sequences in a 'walk' of the viral genome. This can be achieved either in a comprehensive manner or aided by using RNA structure predicting programs to target regions lacking significant secondary structure. In this manner typically from 5 to 30 antisense ODNs thus designed are tested for gene suppression using a range of criterion including *in vitro* translation inhibition, plaque assays, cell viability or proliferation assays, marker gene activity from cells tranformed with viral constructs or by antiviral activity *in vivo* [3].

Sequence specificity, a hallmark of antisense drug design, is evaluated using a range of control oligonucleotides that may include random, sense, missense or scrambled orientation sequences [7]. Using this approach, a number of genes from viruses including HIV, hepatitis, influenza and CMV have been studied as potential targets for antiviral antisense activity (Table 1).

HIV-1

The advent of antisense oligonucleotide technology coincided with the clinical awareness of the human immunodeficiency virus HIV. Thus elements of the HIV-1 RNA genome; gag, rev and tat were identified and investigated as targets for antisense therapy [8, 9]. In early studies using chronically infected, cultured T-cells, incubation with PS antisense directed against the rev and gag genes revealed that there was a sequence-specific inhibition of viral replication [8,9]. However a number of antisense molecules directed to the splice acceptor of the tat gene failed to suppress HIV replication, even after long-term treatment in culture [9]. Along with sequence specific antiviral activity, the antisense ODN have also been shown to have multiple non-sequence specific effects including inhibition of virion binding to the surface to host cells [10]. Further ODN modification using the phosphorodithioate backbone in homopolymers of oligodeoxycytosine produced potent non-sequence specific inhibitors of reverse transcriptase, gp120-CD4 mediated syncyta and HIV replication [11].

Table 1. Antiviral Antisense Oligonucleotides

Virus	Target genes	Chemistry	Assays	Delivery	References
HIV-1	gag	PS, (MBO) PS-/2'-O-Me (MBO) PO-/2'-O-Me imidazole/-NH,	MT-4, Molt 3, M9, U937 cells	DMRIE	[17] [13,14] [16]
	rev	PS	MOLT-3 cells	free	[8,9]
	tat	PS	MOLT-3 cells	free	[9]
HPV	E1	PS	Human foreskin xenograft	free	[18]
	E6/E7	PS	Cervical tumor xenograft, Caski, Siha cells	free (osmotic pump), DOTAP	[19-21]
HBV	HBx	PS	Mouse hepatocellular carcinoma	free	[23]
DHBV	DNA-polymerase	2'O-(2,4-DNP)	duck	free	[26]
	pre-S	PS, PO	duck	free, liposome, PEI	[24,25,29,30]
HCV	5'-NTR	PS, α-PO, β-PO, 2'-MOE + Cm	northern blot - HRA417c cells, luc vector - CV1cells and mouse, in vitro translation	free, DOTMA-DOPE Lipofectin	[33,35-38]
	Core coding region	PS, 2'-MOE	northern blot - HRA417c cells, luc vector - HepG2 cells, in vitro translation	free, DOTMA-DOPE Lipofectin	[34-36]
Influenza A	PB1	PS	MDCK, Clone 76 cells, mouse	TFx-10	[43-45,48]
	PB2	PS	MDCK, Clone 76 cells, mouse	TFx10,DOTAP, DMRIE-C, FuGENE	[43-45,47,48]
	PA	PS	MDCK, Clone 76 cells, mouse	TFx-10	[43,44,46,48]
	NP	PS	MDCK, Clone 76 cells, mouse	TFx-10	[43,44,48]
CMV	IE-RNA	PS	U373 cells, primary human dermal fibroblasts, immunoassay, northern blot	lipofectin	[50-52]

MBO= mixed backbone, PS= phosphorothioate, PO= phosphodiester, DNP= dinitrophenyl, 2'-O-Me= 2'-O-methylribonucleotide, luc= luciferace, Cm= 5-methylcytidine

The early focus on HIV as an antisense target resulted in some of the earliest clinical trials using this antigene approach. In the early 1990s human clinical trials began on the development of Hybridon's 25 mer antisense ODN (GEM91) directed to the HIV-1 gag gene [12]. In cell culture studies, this antisense was shown to have both sequence specific and non-specific anti-HIV activity, including the stimulation of RNAse H cleavage of the gag region and synergy with conventional reverse transcriptase inhibitors [12-14]. Phase I trials indicated that GEM91 was well tolerated at doses up to 4.4 mg/kg/d for 8 days. However, in phase II trials where the drug was administered over 14 days at 3.2 mg/kg a considerable decrease in platelet levels was measured in 3 patients. Combined with the observation of inconsistent responses to treatment, the trial was halted [15]. In more recent studies, "second-generation" anti-HIV antisense molecules have been in development with a range of mixed backbone (MBO) ODNs. These MBOs include the 2'-*O*-methyl- [16], imidizole and primary amine- modified nucleotides [17], combined with the PS or PO backbone. Such modifications have been developed to reduce the inherent toxicity of the PS backbone while maintaining their antiviral activity and have been shown to enhance interaction with RNA and improve pharmacokinetic and pharmacological profiles [16]. The therapeutic potential of these new generations of anti-HIV antisense awaits further clinical development.

HPV

Human papilloma viruses are small DNA viruses that infect mucosal cells with subtypes associated with conditions ranging from genital warts (HPV-6, HPV-11) to cervical cancer (HPV-16 and HPV 18 [18,19]. The polycistronic mRNA coding for the E6/E7 regions from HPV 16 and 18 has been associated with maintaining the malignant phenotype in these subtypes and has been investigated as targets for antisense therapy. Recent ODN/mRNA hybridization kinetics studies have revealed that while few sites in the E6/E7 region are favorable for antisense activity, identification of fast annealing sequences that correlate with antiproliferative activity in HPV expressing cell lines have been demonstrated [20]. Cell proliferation assays were used demonstrate the antiproliferative effects of a 27-mer PS antisense directed to the ATG translational start region of HPV-16 E6 and E7 sequences in both CaSki and SiHa cell lines and primary cervical tumor explants [21]. The specificity of these sequences was confirmed by demonstrating that random PS ODNs had no effect on the proliferation of any of these cell lines and that the anti-E6 and E7 antisense had no effect on the proliferation of endometrial or ovarian tumor primary cell lines.

A subcutanious cervical cancer-murine xenograft model was used to determine the *in vivo* effectiveness of a 16-mer PS anti-E6 ODN that was effective in inhibiting cell proliferation in tissue culture. After 2 weeks of anti-E6 ODN delivery via a subcutanious implanted osmotic pump, growth of tumors treated with anti-E6 was inhibited 70% compared to vehicle or control ODN treated tumors [19]. Antisense ODN directed against the E1 start region of a genital wart forming HPV11 strain was tested for anti-tumor activity in a human foreskin-xenograft model, using donor foreskin slices infected with either HPV11 or a non-homologous HPV-40 virus. The 2'-*O*-methyl modified MBO was effective against cyst formation in both subtypes and subsequent study suggested that an imunostimulatory CpG motif in the ODN was responsible for much of the antiproliferative activity [18]. Such non-specific antiviral activity emphasizes the potential complexity of the mechanism of action of antisense antiviral agents.

HBV

Chronic infection by hepatitus B virus can be a major cause of liver cirrhosis and

hepatocellular carcinoma [22]. Few experimental animal models are available and thus surrogate animal models have been used to investigate the potential efficacy of anti-HBV ODN. A hepatocellular carcinoma model using a mouse transgenic for the hepatitis virus BHx gene was used to study anti-HBx PS ODN efficacy [23]. In this study sequence specific inhibition of both HBx gene expression and the development of prenoplastic lesions were observed after thrice weekly administrations of anti-HBx. More commonly used however is the duck hepatitis B virus or DHBV model. Using this model Offensperger et al. [24,25] demonstrated that a 17-mer PS ODN directed to the 5' region of the pre-S gene to strongly inhibited the viral replication and gene expression in of DHBC both in primary duck hepatocytes and *in vivo* in the Peking duck. To overcome potential toxicity specific to PS ODN, Xin and Wang developed a poly-2'-*O*-2,4-dinitrophenyl modified oligoribonucleotide targeted to the DHBV DNA polymerase gene [26]. In addition to binding sense RNA, these molecules also readily hybridized to the genomic sense DNA and were potent non-sequence specific inhibitors of DHBV reverse transcriptase activity. DHVB-infected ducks treated with poly-DNP-RNA (1 mg/kg/d, 25 d) remained completely viremia free 10 months post treatment.

A number of investigators have been developing formulations to improve the hepatocellular delivery of anti-DHBV-ODN. A multi-component complex of ODN, N-acetylglucosamine-modified BSA, streptavidin and poly-L-lysine has recently been employed to efficiently deliver anti-DHBV ODN to cultured hepatocytes and using this complex, DHBV replication was effectively inhibited *in vitro* [27]. More recently, cationic polyethylenimine (PEI) derivatves have been used to form complexes (20-60 nm diameter) to both stabilize and deliver unmodified PO using the anti-pre-S ODN sequence of Offensperger et al. in cultured cells and *in vivo* [28,29]. This complex was shown to deliver fluorescent-labelled PO ODN into both cultured hepatocytes and into duck liver after intravenous administration [28,29]. The PEI-anti-pre-S PO ODN complex was intravenously delivered at up to 3 mg/kg/d for 9 days and significantly reduced both viremia and intrahepatic DHVB replication in duck liver [29]. Non-complexed PO anti-pre-S ODN had no effect on virema or viral replication. Others developed liposome-encapsulated anti-pre-S formulations that stabilized the PS ODN in the plasma after intravenous injection and improved antisense delivery into the liver [30]. The liposomal antisense was however no more effective than the free ODN at reducing viremia and inhibiting viral replication and in addition may have masked some non-sequence specific antiviral effects of the PS ODN.

HCV

Hepatitus C virus (HCV) is a leading cause of chronic liver disease and like HBV can lead to cirrhosis and hepatocellular carcinoma. HCV also lacks an animal model for study of antiviral agents. The HCV viral genome contains a 5'-non coding region (5'-NCR) that is highly conserved amongst the 6 major genotypes and preceeds the coding region for the polyprotein precursor of this virus [31]. As a result a number of surrogate expression vectors were developed which combine the 5'-NCR (324-341 nucleotides in length) and parts of the adjacent core coding region and a luciferase marker gene system, thus providing a mechanism for quantifying antiviral activity [32]. Using luciferase expression vectors, RNAse H cleavage assays and *in vitro* translation assays investigators identified a few key PS antisense susceptible regions in the 5'-NCR and elements of the core coding region [33-36]. Antisense directed to the region of sequence that included the AUG initiation codon (located at the 3' end of the NCR) had the greatest anti-translational activity. A small-animal model to assess anti-HCV ODN *in vivo* was developed by Zhang et

al. [37] by creating a recombinant vaccinia virus vector (VHCV-IRES) containing the 5'-NCR region and a luciferase marker. This construct produced high levels of luciferase activity in the livers of Balb/c mice which where inhibited by antisense directed to the AUG region of the construct in a sequence specific and dose dependant manner.

A range of chemical modifications were assessed to determine if the specificity of the anti-HCV antisense to the AUG region could be optimized. Using both luciferase expression in HepG2 cells and an *in vitro* translation assay benzylphosphonate- modified ODN had greater anti-HCV inhibitory than either PS or methylphosphonate-modified ODN [38]. A screen of the altered HCV-core protein expression levels in cells treated with range of 2'- modified ODN (2'-MOE, 2'-*O*-methyl, 2'-*O*-propyl, 2'-fluoro) revealed the feasibility of producing translation-inhibiting antisense as this modification inhibits RNAse H activity [36,39]. The 18-mer PS anti-AUG antisense ISIS 14803 was modified to contain five 5-methylcytidine nucleotides that were used to both enhance the RNA binding affinity of the ODN and decrease the immunostimulatory affects of the CpG motifs in the sequence. More significantly, ISIS 14803 (HepaSense) has been shown to reduce patient,s HCV titers in clinical trials [40].

Influenza

Influenza viruses are the cause of acute respiratory disease in humans and a wide range of animals. While prevention against infection has been successful, the constant genetic changes in the surface proteins haemagglutinin and neuraminidase have meant that new vaccines must constantly be derived. Given the highly conserved regions of the polymerase (PA, PB1, PB2) and nucleoprotein (NP) genes of the influenza genome provides number of potential broad range antisense targets. Early work using MDCK cell plaque assays demonstrated sequence specific antiviral activity through a specific effect on the target genes was not determined [41,42]. Takaku's laboratory later developed a transient influenza gene expression assay by sandwiching a chloramphenicol acetyltransferase (CAT) gene between the 5' and 3' terminal sequences of influenza A/PR/8/34 RNA segment 8 RNA from this expression vector was transfected into clone 76 cells into which oligonucleotide was delivered using a cationic lipid complex [43]. Using either CAT expression or cell viability assay as a measure of antiviral activity, antisense ODN directed against PB2 was consistently found to be the most potent inhibitor of influenza replication and gene expression [43-45]. Anti PB1, PB2 and PA antisense were administered intravenously as a cationic lipid complex with Tfx-10 or DMRIE at twice daily intervals 1 day prior to, and 4 days post-infection in a murine infection model with 100 LD_{50} of influenza A/PR/8/34 [46-48]. Anti-PB2 ODN, which is targeted to the AUG region of the PB2 gene, consistently increased both the mean survival time and survival at day 14 post-infection (45%). Concurrent with increased survival was a decrease in PB2 mRNA expression and viral titer measured in infected lungs 4 days after infection.

CMV

Cytomegalovirus exists as a widespread latent virus that is primarily associated with causing severe retinitus AIDS patients [49]. A significant breakthrough occurred with the development of Fomiversen (ISIS 2922), an anti-CMV 21-mer PS ODN complementary to the mRNA of human CMV DNA polymerase gene located in the major immediate early-region [50,51]. The sequence-specific antiviral activity was measured in cell culture using both a 96-well immunoassay and in mRNA expression assays [51]. The mechanism of action of Fomiversin however appears to be complex, including both sense and antisense effects and inhibition of the CMV adsorption to the host cells [51, 52].

Pharmacokinetic studies of Fomiversen injected intravitreally in rabbits and monkeys revealed that the the drug could be detected in the vitreous and retina Accumulation of the ODN in these tissues was dose dependant. After administration, the drug was extensively metabolized to chain shortened intermediates (n, n-1, n-2, etc) and the parent drug could be detected up to 7 but not 14 days after administration [53,54]. Fomiversen is the first antisense ODN drug to be approved use in humans. It is currently being used for second-line treatment of CMV in cases where the patients are intolerant or unresponsive to previous treatments of first line drugs such as foscarnet, ganciclovir or cidofovir [55,56]. This represents a significant milestone in the development of antisense ODNs as an antiviral agent.

B. Gene Suppression with Catalytic DNA

As described above, the most common mechanism for oligonucleotide based suppression is heteroduplex mediated induction of RNaseH which digests the RNA component of the hybrid leaving the DNA to bind to other target molecules. In an alternative strategy, catalytic RNA such as the hammerhead ribozyme (see section C), which have their own built-in RNA cleavage activity can be used to bind and destroy target RNA. These have the advantage of being independent of RNaseH, however, they lack the natural chemical and biological stability possessed by the antisense DNA ODN. While it is possible to manufacture biologically active RNA, its relative fragility in this environment makes it difficult to administer in a direct delivery mode. For this and other reasons, most ribozyme applications rely on transgenic production of RNA *in vivo* within the context of a gene therapy. Advances in nucleic acid chemistry which allow the incorporation of modified ribonucleotide analogues with enhanced nuclease resistance, have led to further progress in ribozyme synthesis [57,58]. Typically, this involves substitution of the 2'-hydroxyl of the ribose moiety with some other chemistry such as 2'-deoxy, 2'-*O*-methyl, 2'-amino or 2'-fluro derivatives. Unfortunately 2'-modification at conserved positions in the ribozyme reduce or eliminate its catalytic activity and thus cannot be applied to the entire oligonucleotide [59-62]. As a compromise between activity and stability, a chimeric composition of normal and modified ribonucleotides has been shown to be feasible [63,64]. Although better than unmodified RNA, these chimeric ribozymes retain some vulnerability to endoribonucleases. By comparison, antisense DNA ODNs with their uninterrupted DNA composition, have a much greater half-life *in vivo*. This natural biological stability of DNA compared with RNA can also be readily supplemented by chemistry that provides even greater resistance to nuclease digestion. However, despite being more suitable for direct delivery, antisense ODNs are devoid of endogenous RNA cleavage activity and thus can only act as passive inhibitors of translation machinery through their sequence specific binding activity, or by generating a heteroduplex substrate for RNaseH mediated decay of the target.

An ideal oligonucleotide based gene inactivation agent targeting RNA would combine the self sufficient activity of ribozymes such as the "hammerhead" and the "hairpin", with the biological resilience of the antisense DNA or ODN. Unlike ribozymes, there is currently no known examples of catalytic DNA in nature, possibly as it is usually locked in a double helical structure which while suited for storage and transmission of genetic information, provides little opportunity for the expression of conformations which might enhance biological reactions such as RNA cleavage. Despite the lack of naturally occuring catalytic DNA, some have recently been derived in the laboratory by artificial evolution systems known as *in vitro* selection [65-68]. In an in *vitro* selection system, single stranded DNA free of its complementary strand is able to

explore alternative structural forms, some of which are capable of catalytic activity, including site specific RNA cleavage and ligation [69,70].

In Vitro Selection

In vitro selection is achieved by combining molecular diversity with the ability to preferentially amplify molecules with a selected characteristic. Molecular diversity of the selection pool is established by degenerate oligonucleotide synthesis, while amplification of the construct and selected molecules is facilitated by PCR. Selection occurs through partitioning molecules that possess some of the selected attribute from those in the vast majority which are deficient. In the case of RNA cleaving DNAzyme selection, active molecules can be separated from their inactive counterparts by the difference in their respective molecular weights (after self cleavage of an imbedded RNA) using gel electrophoresis. Prospective catalysts of this type can also be attached to a solid support *via* their substrate sequence, from which only molecules with RNA cleavage activity are released.

The possibility of an RNA cleaving catalytic DNA was initially explored in selection constructs containing mostly single stranded DNA with a single embedded ribonucleotide for substrate. Downstream from the substrate, a section of randomised sequence (40-50 bp) provided molecular diversity, while fixed sequences on each flank provided stable sites for primer binding. A sample population from these combinatorial libraries was immobilised on a solid support containing streptavidin via a biotin tag (Fig. 1). After washing and stripping the complementary template strand in alkali, a buffer containing an appropriate divalent metal ion (Pb^{2+}, Zn^{2+}, Mn^{2+} or Mg^{2+}) was introduced to generate conditions conducive to a *cis*-cleavage reaction [65,66]. The release of active members in the library by this self-cleavage allowed them to be selectively amplified and regenerated by PCR for subsequent rounds of selection. After numerous cycles of selection and amplification the self-cleavage activity of the remaining population was examined by electrophoresis of an end labelled fraction which was allowed to fold and react in solution. When it was established that the selected pool contained a satisfactory level of self cleavage activity, the PCR products were cloned and sequenced. The cloned sequences were then used to examine the cleavage activity of individual molecules and predict secondary structures. The secondary structure predictions for the intramolecular configuration of each enzyme-substrate complex were then used to separate these two components of the *cis* arrangement, such that the enzyme and substrate would interact in *trans*. More general *trans* cleavage capacity of these DNAzymes was then examined using separate oligonucleotide enzyme and substrates. Using this selection protocol, in the first instance with Pb^{2+} and then with other buffer systems including Mn^{2+}, Mg^{2+} and Zn^{2+}, different DNAzymes were found which could utilise each of these divalent metal ions to cleave a sequence containing a single embedded ribonucleotide [65,66]. In the case of the magnesium dependent DNAzyme this was accomplished with a rate up to 10^5 times that of the uncatalysed reaction.

After further development of the *in vitro* selection protocol, Santoro and Joyce [67] evolved Mg^{2+} dependent DNA enzymes capable of cleaving biologically relevant, all RNA substrates. From this selection experiment two prototypes were characterised and denoted the "8-17" and the "10-23" RNA cleaving DNA enzymes (Fig. 2). Both of these molecules are reminiscent of the hammerhead ribozyme in that they contain conserved catalytic domains flanked by variable binding domains. Each binding domain was originally evolved to specifically complement the substrate sequence used in the selection protocol, however, as this was achieved in both of these DNAzymes through

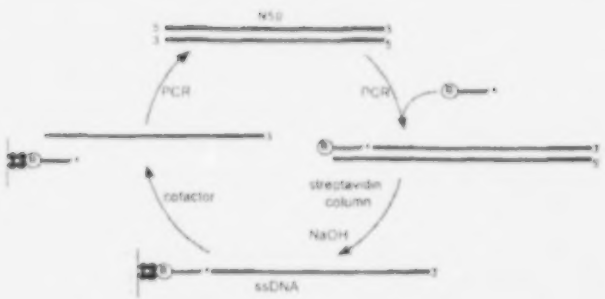
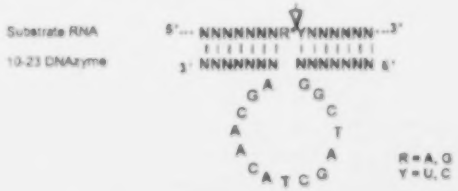


Figure 1. A selection scheme for the evolution of RNA cleaving catalytic DNA Adapted from [10]. See text for detail. In this system a combinatorial library of different DNA sequences is generated by the synthesis of an oligonucleotide template with a 50 base (N50) stretch of random sequence (hatched lines). To enable amplification and reselection, each molecule from this diverse pool contains a fixed primer binding sequence at each end (black). Immobilisation is achieved by tethering this assembly of potentially functional sequences through a biotinylated (B) RNA containing substrate (rA)to a solid streptavidin-coated support. After alkali denaturation (to strip away the template strand) the single stranded residue is allowed to fold around its substrate linkage in the presence of suitable buffer and cofactors. The eluent of this washing (which contains catalytically active molecules) is then used as a PCR template for regenerating the assembly for a second round of selection. After multiple rounds of selection the relative abundance of each active molecule should be high enough to visualise the activity in a cleavage assay and clone the DNA for sequencing.



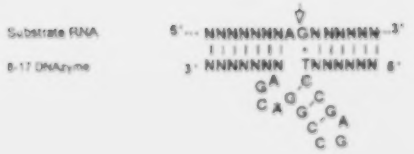


Figure 2. Secondary structure of the "10-23" and "8-17" general purpose RNA cleaving deoxyribozymes and paired substrate Panels A and B contain illustrations of the 10-23 and 8-17 deoxyribozymes respectively. Watson-Crick interactions for each deoxyribozyme-substrate complex are represented by generic ribonucleotides (N) in the target (top) and the corresponding deoxyribonucleotides (N) in the arms of the deoxyribozyme (bottom) of each. The defined sequences in the loop joining the arms and spanning a single unpaired purine at the RNA target site of each model represent the conserved catalytic motif.

Watson-Crick base pairing, the possibility existed that the specificity could be altered to suit any target RNA sequence. This was particularly successful in the 10-23 DNAzyme, which was found to have a minimum sequence requirement of a purine-pyrimidine pair at the site of cleavage so long as flanking base pairing was maintained. Despite being less flexible, the 8-17 DNAzyme with its requirement for an AG in the substrate, can also be described as a general-purpose endoribonuclease [67]

The "10-23" RNA Cleaving DNA Enzyme

The 10-23 DNA enzyme or DNAzyme was named from its origin as the 23rd clone characterised from the 10th cycle of *in vitro* selection [67]. This enzyme has a number of features, which endow it with tremendous potential for applications both *in vitro* and *in vivo*. These include its ability to cleave almost any RNA sequence with high specificity provided it contains a purine-pyrimidine dinucleotide. This can be accomplished at very high kinetic efficiency with rates approaching and even exceeding those of other nucleic acid and protein endoribonucleases [67]. This activity is all the more remarkable when considering that it is achievable at concentrations of magnesium in the physiological range. After characterisation of the 10-23 catalytic motif, more rounds of reselection and amplification were undertaken with a partially randomised sequence (25% degeneracy) with the aim of further optimising the catalytic activity [67] Surprisingly, this selection did not yield any refinement of 10-23 DNAzyme, indicating that the catalytic motif was highly evolved and had very little sequence redundancy.

Catalytic Activity of the 10-23 DNAzyme

As the 10-23 DNAzyme is capable of catalysing the hydrolysis of RNA at purine pyrimidine junctions, the AUG initiation codon of any gene can be used as a target. Early kinetic analysis of the 10-23 DNAzyme focused on synthetic substrate sequences derived from the start codons of various HIV genes [67]. A feature that emerged from this analysis was that the kinetic efficiency of DNAzyme catalysed cleavage varied substantially between different substrates. This sequence dependent variability appeared to be closely associated with the thermodynamic stability of the enzyme-substrate heteroduplex as predicted by the hybridisation free energy [71]. In this relationship, DNA enzymes with the greatest heteroduplex stability indicated by a low free energy of hybridisation (calculated using the nearest neighbour method), was often found to have the greatest kinetic activity. The sensitivity to heteroduplex stability in many cases can be counterbalanced by increasing the DNAzyme arm length until the hybridisation free energy decreases to the optimal level. At this point the enzyme activity can approach its maximum efficiency. Other factors that effect heteroduplex stability include the general GC content and specific pyrimidine content of the DNA [71-73]. The influence of heteroduplex stability on the kinetic efficiency of the DNAzyme is probably derived from its effect on the K_M of the reaction. The inverse relationship between K_M and enzyme-substrate complex stability can be observed by increasing the substrate binding domain length such that the heteroduplex stability is increased which usually causes the K_M to fall towards a minimum. The benefit to the overall kinetic efficiency obtained by increasing binding domain length, however, is limited by the adverse effect it has on catalytic turnover (indicated by the k_{cat}) which occurs when the enzymes increased affinity for the products slows down the catalytic cycle by reducing the rate of product release. In more recent investigation of this behaviour in reactions where the substrate-binding domain length ranged between 4/4 (base length/arm) to 13/13, the maximum overall efficiency (k_{cat}/KM) under physiological reaction conditions was found with an arm length of between 8-9 bp [68]

Most kinetic analysis of the 10-23 DNAzyme has been performed on molecules

with symmetric RNA binding domains irrespective the contribution of each arm to the total thermodynamic stability of the enzyme-substrate complex. There is some evidence to suggest to that the optimal design of a 10-23 DNAzyme for a given target site, should take into account both the sequence and length of the individual binding arm to accommodate the differences in stability of their respective complexes with the substrate. Experiments with different length binding domains, have shown that in some instances the rate of DNAzyme catalysed cleavage, can be enhanced by asymmetric arm length truncation. In the case of DNAzymes targeting the c-myc translation initiation region, we found that the observed reaction rate was highest in the molecule with a binding arm length ratio (5′/3′ bp) of 6/10 [74]. This relative advantage derived by truncation of the binding domain on the 5′ side of the catalytic domain was similar to that seen in the hammerhead ribozyme which had an optimal length ratio (helix I/helix III) of 5/10 [75]. The benefit of a shorter helix I in the ribozyme was attributed to a possible decrease in counterproductive interference between it and helix II. In the case of the DNA enzyme, which lacks the equivalent of a helix II, this effect is more likely related to the respective heteroduplex stability of each individual binding domain-substrate complex. In support of this argument, the hybridisation free energy for the truncated 5′ arm of the c-myc cleaving DNAzyme was found to be unusually low indicating that it was capable of generating more stability than expected for its size compared with the average duplex sequence. However, while this explains the fact that there was no reduction in rate compared with the longer symmetric counterpart, it does not really explain why there was an apparent rate enhancement in the shorter molecule. One possibility is that when this RNA substrate is bound with exceptionally high stability by the full-length DNAzyme, the reaction rate is inhibited by the rigidity of the enzyme-substrate complex. Another possibility is that the full-length DNAzyme is capable of forming an alternative secondary structure, which is not catalytic.

In contrast to the observations in this system, truncation of the same arm from a DNAzyme targeting a different substrate derived from the HPV16 E6 translation initiation region, resulted in a decrease in reaction rate. In this case the predicted hybridisation free energy of the 5′ arm was substantially higher, which may have made it more sensitive to shortening than the previous example.

RNA Sequence Specificity

With the potential to bind any RNA sequence and cleave purine-pyrimidine junctions, the 10-23 DNA enzyme has unprecedented target site flexibility. However, despite the enormous capacity to cleave different sequences, the actual substrate specificity of an individual DNAzyme with defined RNA binding domains, appears to be very high. This ability to discriminate is particularly important in biological applications where unwanted side reactions between the DNAzyme and some closely related or unrelated substrate could be very undesirable. The issue of specificity was central in a study by Taira and coworkers comparing the *in vitro* cleavage activity of DNAzymes and hammerhead ribozymes targeting RNA derived from the junction of the *bcr-abl* fusion [76]. In this system DNAzymes had the advantage for two reasons; one, they were able to get closer to the junction because of their superior target flexibility and two, their activity was more easily perturbed by mismatch and hence were less reactive with the RNA sequence from the wild type *abl* gene. The difference between the ribozyme and DNAzyme in this study was attributed to the lower stability of the DNA-RNA heteroduplex compared with the RNA-RNA homoduplex [77]. Indeed when the heteroduplex stability was increased by lengthening the RNA binding domains, the specificity of the *bcr-abl* (b2a2) cleaving

DNAzymes also decreased slightly [78] In order to achieve the desired specificity in the *bcr-abl* (b3a2) target, Wu et al utilised DNAzyme arm length asymmetry (6/12 bp), such that the short arm less likely to allow cleavage of *bcr* transcripts [79]. However, the key point is not so much the difference in duplex stability, but the sensitivity to this difference displayed by the DNAzyme because it maintains relatively high activity at comparatively low heteroduplex stability. However, when the stability falls below a threshold level due to the introduction of a binding domain mismatch or truncation, the catalytic activity of the molecule can become severely impaired. The influence of single base mismatch was demonstrated empirically at different positions of the substrate by the introduction of point mutations [68]. In this analysis, any mismatch with the substrate was detrimental to the catalytic efficiency, although the extent of this effect varied substantially from one position to the next and between different types of mispairing. We have also examined the specificity of the 10-23 DNAzyme by observing its ability to discriminate between sequences which differ by as little as a single nucleotide polymorphism [80]. In this experiment, reactions between DNAzyme and matching substrate sequences (derived from a polymorphic site in the L1 gene of 6 different clinically relevant HPV types) were compared with reaction in the unmatched substrates. In each case only the perfectly matched type-specific DNAzymes were capable of achieving substantial cleavage of the corresponding substrate despite the similarity between the different sequences. In each of these studies the specificity of cleavage was examined with respect to binding domain-substrate interactions where some mismatches, particularly those producing a "wobble" pair, can be tolerated [68,80]. If however, the difference between the target and non-target substrate lies at the cleavage site, such that the purine-pyrimidine (R-Y) becomes R-R, Y-Y or Y-R, then the DNAzyme would have no activity on the non-target substrate.

Antiviral Activity of 10-23 DNAzyme

The ability of the 10-23 DNAzyme to specifically cleave RNA with high efficiency under simulated physiological conditions has given rise to expectation that this agent may have useful biological application as an antiviral agent. To explore this potential a number of groups have attempted to examine the activity of DNAzymes against a number of pathogenic viruses (summarized in Table 2 [81-95]) In our laboratory we initiated experiments with DNAzymes targeting the sequences from the HPV 16 E6 and E7 genes. These molecules designed against the translation initiation regions were optimised in terms of RNA binding domain length through kinetic analysis of *in vitro* RNA cleavage reactions. To improve the stability of the DNAzyme oligonucleotide to serum and intracellular nucleases whilst maintaining catalytic activity, a single 3′-terminal nucleotide inversion modification was introduced to the oligonucleotides during synthesis by the formation of a 3′-3′ internucleotide linkage. After ensuring the catalytic activity was preserved, the serum nuclease stability of the modified oligonucleotides was examined by incubation in human serum. In this environment oligonucleotides modified by 3′ inversion were found to have a half-life of 24 hours at 37°C compared with 2 hours with the unmodified counterpart. The modified anti-E6 DNAzymes were cotransfected with an E6 gene expression vector into 3T3 cells with aid of the DOTAP transfection reagent. In this transient E6 expression system the anti-E6 DNAzyme was found to substantially reduce the level of E6 mRNA compared with a non active oligonucleotide with the same nucleotide composition (unpublished observations).

Banerjea and coworkers have examined the activity of several 10-23 DNAzymes targeting both the HIV and associated host factors in various model systems [83,85,87,89,90]. In early work a luciferase reporter construct is used to indicate the extent of HIV-1 envelope-CD4 mediated cell fusion [96] DNAzymes.

Table 2. Summary of virus related DNAzyme gene suppression studies

Target	Cell	Arms	Modification	Delivery	Activity	Controls	References
HPV E6	3T3 (transient)	8/8	3'- inversion	DOTAP	60% suppression E6 RNA	scrambled	unpublished
HIV-1 env	HeLa (transient)	7/7	none	Lipofectin	50% fusion	irrelevant & antisense	[83]
CCR5	HeLa (transient)	7/7	none	Lipofectin	50% fusion	irrelevant	[85]
CXCR4	U87	7/7	none	Lipofectamine	80% fusion	irrelevant	[87]
HIV-1 gag	COS-1 (transient)	7/7	none	Lipofectin	80-90% suppression	inactivated	[88]
HIV-1 tat/rev	THP-1, Cos-1 & HeLa	7/7	3' poly G	Lipofectin	80% suppression pNL Luc reporter gene expression	inactivated	[89]
HIV-1 tat/rev&tat	Cos-1 & HeLa	7/7	dimer DNAzyme	Lipofectin	60-80% suppression pNL Luc reporter gene expression	inactivated	[90]
HIV-1 env	U87	7/7	none	Lipofectamine	77-81% suppression viral load	inactivated	[84]
HCV 5'NCR	Huh 7 transient	15/15	2+2 PS cap	Lipofectamine	50% inhibition	inactivated	[93]
HBV X gene	HepG2/COS-1	7/7	none	Lipofectin	75% inhibition	inactivated	[94]
InfluenzaA PB2	MDBK/MDCK	7/7,8/8	none	Lipofectin	>90% inhibition	random	[95]

which cleave the HIV *env*, CCR5 chemokine receptor and CXCR4 chemokine receptor transcripts were all found to be potent inhibitors of fusion between CD-4 positive HeLa cells and HeLa cells expressing the HIV-1 envelope gene. More recently the plasmid encoded HIV replication system pNL4-3 incorporating a luciferase reporter gene has been used in a number of studies from the same group to determine the influence of anti-HIV DNAzymes on both HIV-1 gene expression (gag, tat, and tat/rev) and replication [88-90]. In the first of these reports a library of DNAzymes containing randomised binding arm sequence was used to detect DNAzyme with cleavage activity against a gag transcript. DNAzymes found through this process were then used in the biological assay and found to have anti HIV activity [88]. In another study an anti gag DNAzyme was modified by an 11-G extension at the 3' end with the intension of enhancing the oligonucleotides tropism for macrophages [89]. This addition was found to increase macrophage specific uptake of naked oligo, possibly by binding to the scavenger receptor in these cells. In an alternative configuration of this DNAzyme, short polyG and polyC tracts on the termini were used to generate stem-loop structures with the aim of protecting the oligonucleotide from degradation. Although successful at improving the stability in medium supplemented with serum, these structures were found to be detrimental to the enzymes RNA cleavage activity and biological activity. In the third study a combination DNAzyme designed to cleave sites in both the tat and tat/rev RNA was examined [90]. Unfortunately, the long dimer-DNAzymes ability to cleave both target substrates and suppress HIV was reduced compared to the individual free DNAzymes - possibly due to the additional secondary structure constraints.

HIV was also used as a target for DNAzymes by another group [84]. From a

series of nine different DNAzymes directed to the conserved V3 loop of HIV *env*, the one selected on the basis of its superior cleavage activity *in vitro* was found to be capable of suppressing both viral replication (viral load/p24 antigen) and virus infection (single cycle luciferase reporter system).

The 10-23 DNAzyme has also been shown to be active against viral mRNA targets from the Hepatitis C and B, and was able to protect cells from infection with influenza A virus [93-95]. Hepatitis C non-coding region-luciferase fusion mRNA expressed in Huh7 cells was found to be sensitive to two DNAzymes sites tested. In both cases the biological activity was enhanced by the phosphorothioate modification of two terminal linkages and by increasing the binding domain lengths from 11 to 15 bases. Mutant DNAzyme lacking cleavage activity retained some biological activity, probably as a consequence of an antisense effect. This is perhaps not surprising given the unusually long arm lengths that could each support ribonuclease H activation. Hepatitis gene X expression in HepG2 and Cos-1 cells was also found to be sensitive to specific monomer and dimer DNAzyme co-transfected with the target gene X expression vectors [94]. As observed in other studies, however, there was no clear advantage to the dimer approach compared to the monomer DNAzymes. In another study, a DNAzyme targeting the translation initiation region of the PB2 gene of influenza A was found to suppress viral replication in MDBK cells [95]. The in vitro anti-influenza activity of this DNAzyme was found to be superior to an inactivated control and position matched antisense phosphorothioate oligonucleotide. The biological activity of this DNAzyme appeared to also be effected by binding domain length with the longer 8/8 molecule performing better than the 7/7 base analogue at both in vitro cleavage and viral suppression

DNAzyme Target Site Selection

The design flexibility of the 10-23 DNAzyme owes largely to its ability to bind a target RNA sequence via Watson-Crick base pair interactions. However, like other agents that function by hybridisation with single stranded RNA, the DNAzyme must compete with the targets own prevailing intramolecular base pairing which can form stable secondary structure While most mRNA substrates provide many opportunities for DNAzyme cleavage, finding these sites in the target RNA which are amenable to efficient hybridisation and cleavage is usually a difficult and time consuming task involving empirical testing of many DNAzymes in long folded RNA transcript *in vitro*. In order to streamline this process, we developed a multiplex approach to target site selection, which allowed the simultaneous analysis of many different DNAzyme cleavage sites in a single reaction [81]. With this strategy the cleavage efficiency of eighty different DNAzymes targeting the entire E6 component of the full length E6/E7 transcript from HPV16, was determined in a single experiment. Molecules with both efficient and inefficient RNA cleavage activity were then compared for their ability to inhibit E6 gene expression in a cell free system. The DNAzymes designated most efficient cleavers were also the most efficient inhibitors of E6 gene expression The multiplex assay was found to be an effective screen for DNAzyme cleavage sites, as it could efficiently identify the molecules with high activity against long folded substrate RNA DNAzymes with efficient *in vitro* cleavage activity were also shown to be more likely to posses biological activity than DNAzymes with poor cleavage activity. In addition to screening for effective cleavage sites, the multiplex selection assay may also be useful for identifying target site accessibility for other RNA binding agents such as antisense oligonucleotides and ribozymes.

C. Ribozymes as antiviral therapeutic agents

Ribozymes are catalytic RNA molecules possessing at the very least, enzymatic cleavage and ligation activities [97-99]. In nature,

enzymatic RNA molecules catalyze sequence-specific RNA processing The specificity is determined by Watson-Crick base-paring between ribozymes and nucleotides near the cleavage site of the target RNA. By altering substrate recognition sequences, several intramolecular *cis*-cleaving ribozymes have been engineered to cleave target RNA in trans [100] Theoretically, these *trans*-cleaving ribozymes can be designed to cleave any RNA species in a sequence-specific manner. Thus, the mRNA coding any proteins associated with a disease can be selectively cleaved by ribozymes. Consequently, ribozymes have become potentially valuable tools for the inhibition of virus replication, modulation of tumor progression and analysis of cellular gene function

Trans-cleaving catalytic RNAs

At present, there are five major RNA catalytic motifs that are derived from naturally occurring ribozymes: hairpin, hammerhead, group I intron, ribonuclease P and hepatitis delta virus ribozyme. Among those, two RNA catalytic motifs that originate from plant viroid and virusoids have received much attention for their potential use due to their inherent simplicity, relatively small size and the ability to be incorporated into a variety of flanking sequence motifswithout changing site-specific cleavage capacities. These are hammerhead and hairpin ribozymes and illustrated in Figure 3. The hammerhead ribozyme model is based on the satellite RNA strand (+) of tobacco ringspot virus (sTobRV). It has three basic components· (i) a highly conserved 22 nucleotide-catalytic domain, (ii) base paring sequence flanking the susceptible 3′, 5′ phosphodiester bond; and (iii) a recognition sequence on the target RNA such as GUC The cleavage reaction occurs 3′ to the recognition sequence with formation of a terminus containing a 2′, 3′ cyclic phosphodiester and a 5′ hydroxyl terminus on the 3′ fragment 4). The (-) strand of the sTobSV forms a two-dimensional hairpin structure, with four major helical regions and possessing catalytic activity. On the basis of this structure, the hairpin ribozyme model consists of four helices and five loop regions formed between a 50 base catalytic RNA and a 14 base substrate RNA. Helices 3 and 4 are within the ribozyme itself, and the ribozyme binds to the target RNA through helix 1 (six base pairs) and helix 2 (four base pairs), separated by a NGUC loop in the substrate strand. The recognition sequence is bNGUC, where b is G, C or U; N is any nucleotide, and cleavage occurs 5′ to the G residue [98].

Group I introns are derived from the self-splicing intervening sequence of Tetrahymena thermophila. The enzymatic activity of group I introns involves a two-step transesterification with a requirement for a divalent cation, such as magnesium, and a guanosine cofactor The first step involves a nucleophilic attack of the guanosine cofactor 3′-hydroxyl group on the 5′-splice site forming a free 3′-hydroxyl on the 5′ exon In a second step, this free hydroxyl group makes a nucleophilic attack on the 3′ splice site, releasing the intron as a circular molecule and leaving a ligated exon [101]. Substrate specificity is determined by a sequence within the intron, the internal guide sequence (IGS), and there is a requirement for a U at position −1, relative to the cleavage site, paired with a conserved G in the IGS [102].

RNAse P is a ribonucleoprotein involved in processing the 5′ termini of tRNA precursors during their maturation. RNA cleavage is via nucleophilic attack on the phosphodiester bond leaving a 5′- phosphate and 3′-hydroxyl at the cleavage site and there is an absolute requirement for divalent metal ions. The *E coli* RNAse P comprises an RNA domain of ~ 400 nucleotides (the M1 subunit) and a protein component of 14,000 daltons dubbed C5. Both *E. coli* and human RNAse P were shown to cleave a target RNA that resembles a tRNA substrate in a bimolecular reaction [103]. The 3′-proximal sequence of the stem functions to identify the target and is considered to act as an external guide sequence (EGS) which can be targeted to essentially any target RNA

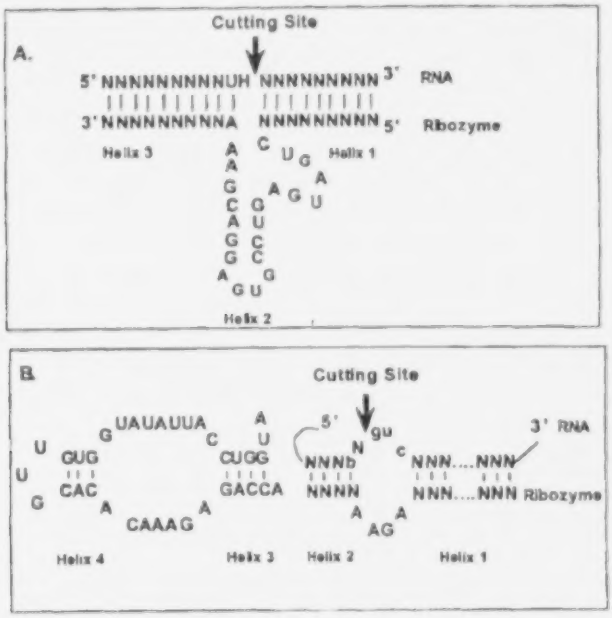


Figure 3. Structures of hammerhead (A) and hairpin ribozymes (B).

The use of ribozymes as antiviral agents

As with all emerging technologies, there are several areas that must be addressed before the technology can be applied for human therapy. Among theses are (1) reaction kinetics; (2) optimum design and (3) target site selection.

Ribozyme kinetics

A large number of studies have been performed to provide a better understanding of the mechanism of ribozyme-mediated catalysis. In general, a minimal kinetic description for one turnover of hammerhead ribozyme reaction involves assembly of ribozyme (E) and substrate (S) into an E•S complex, (2) cleavage of the phosphodiester bond, generating a 5′ product with a 2′, 3′-cyclic phosphate terminus (P1) and a product with a 5′-hydroxyl terminus (P2) that remains bound to the ribozyme, and (3) release of the products. A Michaelis-Menten mechanism has been established by using multiple- and single-turnover conditions for the formation of the ribozyme-substrate complex and its subsequent conversion to products [104,105].

Under multiple-turnover conditions, the substrate is in excess over the ribozyme so that the ribozyme can catalyze the cleavage of several substrate molecules. The catalytic rate constant or turnover numbers, k_{cat}, is a measure of the rate-limiting step, which can be cleavage, conformational transitions of the ribozyme-substrate complex, or product release. When a ribozyme with six bases in each arm was used in kinetic analysis, k_{cat} and K_M were within typical values of 1- 2 min^{-1} and 20–200 nM respectively. Extension of the helical arms generally results in an increased

stability and markedly decreased k_{cat} [106]. Under single-turnover conditions, the ribozyme is in excess over the substrate and such conditions are normally used for cleavage of long mRNA substrate. Cleavage rates are generally several orders of magnitude lower, compared to the rates obtained for short substrate [107]. In the case of hammerhead and hairpin ribozymes, the presence of Mg^{+2} is essential for the cleavage, and the convincing evidence suggested that Mg^{+2} not only assists in RNA folding but also participates directly in the cleavage mechanism [108].

Optimizing design

Although mutational, chemical and physical investigations have advanced our understanding of how ribozymes act in vitro, for a specific target RNA the optimal design of ribozymes for both in vitro and in vivo use still has to be experimentally determined. Here using the hammerhead ribozyme model as an example, some of the basic rules in ribozyme design will be discussed.

Hammerhead ribozymes such as those illustrated in Figure 3, can cleave any 5'-NUH-3' triplets of an RNA, where U is conserved, N is any nucleotide and H can be C, U, A, but not G. This cleavage rule has been further extended from the work of several groups [109-112]. Comparative studies revealed that the reaction rate (kcat) decreases in the following order: AUC, GUC>GUA, AUA, CUC>AUU, UUC, UUA>GUU, CUA>UUU, CUU. Results from recent studies extend the repertoire of cleavage sites for hammerhead ribozyme application to inhibition of gene expression. Kore et al. [113] report that the NUH rule can be modified to the NHH rule, where H is any nucleotide except G. However, whether this extension can be applied to in vivo applications is yet to be tested in a suitable model. This can be further complicated when the target RNA structure in cellular environment is taken into consideration.

In addition to the NHH triplets for cleavage specificity, the ribozyme arm sequence context can also influence cleavage rate significantly. In a simple term, the longer the binding arms, the lower the turnover in cleavage of short substrates. The length of the 3' arm is apparently more critical for specificity than that of the 5' arm [114]. For a long substrate RNA, intramolecular structures of the target RNA could interfere with the binding of a ribozyme, and for in vivo application cellular proteins could also affect ribozyme activity. Results from variations in arm length of ribozymes from 5 nt to 18 nt have indicated that the ribozyme activity is closely related to the arm length both in a symmetric or asymmetric models, and this depended somewhat on the sequence context [115-118].

Target selection

Given that the biophysical principles governing RNA folding in vivo have not been clearly determined, there is no clear means available to determine the accessibility of a potential RNA substrate cleavage site. As a first approximation the gross topography of the substrate RNA can be simulated by analysing the region surrounding the cleavage site using an RNA secondary-structure folding program. In this way, it may be possible to determine whether or not the target site is buried within an obvious thermodynamically stable region of secondary structure. However, there are limitations to the size of RNA that may be analysed by this method and this is clearly a gross approximation of RNA secondary structure that does not take into account tertiary structure nor RNA - protein interaction which may be present in the intracellular context. Other tools for empirical testing of cleavage site accessibility such as S1 nuclease or RNase mapping, can supply additional information [119,120]. As an alternative, an assessment of biological significance may allow one to predict certain features of the RNA that would indicate accessibility. For example, a number of regions in the HIV-1 genome have been targeted by different groups using ribozymes, including the 5' leader region [121,122], *gag*

[123] and *tat* genes [124-126] and the Ψ packaging site [127]. These target sites are generally exposed for potential RNA-RNA or RNA-protein interactions as a result of their biological function, and thus should be potentially accessible in cells.

In addition to enzymatic mapping of accessible sites in RNA, combinatorial approaches may be used to identify ribozyme accessible sites, in which no assumptions regarding the best target sites and corresponding ribozymes are made. This approach may provide a cost-effective mapping technique and could speed up the process of drug discovery. Lieber and Strauss [128] first reported the use of combinatorial approach for the selection of ribozyme cleavage sites. A library of ribozyme genes with random sequences of 13 nucleotides on both sides of the hammerhead was generated. Using RACE technique, they successfully identified cleavage sites, and subsequently re-amplified and cloned the ribozyme genes. In our laboratory, a similar design was employed to construct a synthetic ribozyme library and sensitivity of detection of cleavage sites was increased significantly by using ligation-mediated PCR (Kim et al., unpublished results). More recently, a simpler approach that requires neither PCR nor cloning was also recently reported by Yu et al. [129], in which a population of in vitro transcribed hairpin ribozymes with randomised substrate-binding arms, was incubated with the substrate RNA and cleavage positions were determined by primer extension.

Ribozyme-mediated suppression of gene expression has proven to be neither straightforward nor routine partly due to the difficulty in defining the most accessible sites. *In vitro* screening provides the simplest and most direct approach to identifying the accessible sites although there are some concerns in terms of correlation between *in vitro* and *in vivo* results. However, a combined approach of cell-based screening systems for target site selection and in vitro screening using the full-length mRNA will provide valuable information for the design of gene-targeted ribozymes.

In vitro and in vivo antiviral activities and clinical applications

The gradual maturation of ribozyme technology from the bench to clinical application involves several major challenges, many of which still need to be resolved. These include extra- and intracellular stability of the ribozyme, delivery of ribozymes to target cells, target accessibility, co-localization of ribozyme and target within cells and optimal catalytic activity and specificity of the ribozyme. Despite their enzymatic activity, ribozymes must be delivered to target cells in amounts sufficient to both affect a significant proportion of the cell population and to affect a significant proportion of the target mRNA. While therapeutic application of ribozymes may be achieved by exogenous delivery of chemically produced ribozyme complexed, for example, to a cationic lipid, for certain applications, such as their use in chronic disease or in an antiviral therapeutic settings, they may require permanent availability to be of therapeutic benefit. This can be achieved by transfer of genes encoding for ribozymes using viral vector systems [130].

The use of ribozymes as antiviral agents has been primarily focused on several clinically relevant human viruses, including HBV, HCV, herpesviruses, HIV, HPV and influenza viruses. This is summarised in Table 3. Here, as an example, we will discuss some of the aspects of ribozyme-based gene therapy for AIDS. A simple approach to treating HIV-infected patients is the infusion of transduced and "protected" $CD4^+$ PBLs. If ribozyme the construct can protect $CD4^+$ T cells from HIV-1 infection and its sequelae in patients, then the decline in the numbers of $CD4^+$ T cells could be halted or even to some extent reversed and HIV-infected individuals could benefit clinically. It is relevant to note that the half-life of an HIV infected $CD4^+$ T lymphocyte is of the order of only two days. We have initiated two independent Phase I clinical trials to test safety of this approach, the ability to detect ribozyme-containing cells in the bloodstream and the

Table 3. Summary of ribozyme-based antiviral approaches.

Viruses	Target genes	Ribozymes	Vector	Assay		References
HBV	*Epsilon*	*hammerhead*	*U6 SnRNA vector*	*in vitro/cell extract*		*[132]*
	HbsAg, Pol, X protein	hairpin	Retroviral vector	in vitro/ Huh7 cells		[133]
	HbsAg	RNaseP/EGS		in vitro		[134]
	X protein	hammerhead	plasmid	in vitro/HepG2 cells		[135]
	pg RNA	hairpin		polII & III constructs	HCC cells	[136]
	X protein	hammerhead	EGFP vector	Cells		[137]
	PolyA signal	hammerhead	plasmid	in vitro/HepG2 cells		[138]
HCV	5'UTR/capsid	hairpin	retroviral vector	in vitro/HepG2		[139]
	+/- RNA	hammerhead	adenoviral vector	hepatocytes		[140]
	5'NCR	hammerhead	plasmid	reporter gene assay		[141]
	5'UTR	hammerhead	synthetic	reporter system/mice		[142,143]
Herpesvirus	HSV-1 IE3	hammerhead		in vitro		[144]
	HSV-1 TK	In vitro selected				
		RNaseP	retroviral vector	in vitro/cells		[145]
	CMV IE1, IE2	RNaseP	plasmid	cells		[146]
	HHV8 vIRF	hammerhead	pBI-EGFP	HelaTet-Off cells		[147]
HIV	gag	hammerhead	plasmid	HelaCD4+		[123]
	tat	hammerhead	retroviral vector	Jurkat		[124]
	Leader sequence	hairpin	retroviral vector	T cells		[122,148]
	tat	hammerhead	retroviral vector	T cells/PBL		[125,126]
	ψ packaging region	hammerhead	pSV2neo	SupT1		[127]
	CCR5	hammerhead	retroviral vector	CD34+		[149]
HPV	HPV16 E6/E7	hammerhead	AAV	in vitro	[150]	
	HPV18 E6/E7	hammerhead	plasmid	in vitro	[151]	
	HPV16 E6/E7	hairpin	plasmid	keratinocytes		[152]
	HPV 16 E7	hammerhead	Adenoviral vector	in vitro/mice		Sun et al unpublished
Influenza A	Segment 5	hammerhead/hairpin	pSV2neo	in vitro/COS cells		[153]
	PB1	hammerhead	Adenoviral vector	CV-1 cells		[154]

hypothesis that ribozymes can protect $CD4^+$ T-lymphocytes from rapid HIV-1 mediated destruction within an infected individual. Both trials utilise LNL6 vector (a MoMLV-based expression vector) and recombinant LNL6 containing Rz2 (RRz2), and each trial uses a separate target cell population - $CD4^+$ PBLs and $CD34^+$ stem The first trial involves identical twins, discordant for infection with HIV [155]. Healthy $CD4^+$ lymphocytes from the uninfected twin are transduced with a retroviral vector containing the ribozyme gene. These transduced cells are cultured and expanded *ex vivo* before transfusion into the bloodstream of the corresponding HIV-positive twin. The second clinical trial involves the removal, transduction and transfusion of $CD34^+$ stem cells within HIV-positive individuals [156]. The rationale being that these transduced stem cells will differentiate and give rise to a variety of lineages that express the ribozyme

In each of the trials, separate populations of cells are transduced with the retrovirus vector containing the ribozyme and the vector alone as a control, and equal numbers of these two transduced cell types are then introduced into the recipient patients. This has allowed us to monitor the survival of ribozyme expressing $CD4^+$ lymphocytes relative to a similar

population of transduced $CD4^+$ lymphocytes which have been processed in an identical fashion but do not contain the ribozyme sequence. Cell survival is being monitored by detecting ribozyme and control vector DNA sequences in peripheral blood using quantitative PCR procedures. Preliminary data indicate that transduction of the ribozyme construct into human cells is safe and the transduced cells can be detected in all the patients.

In summary, a large body of work on ribozyme catalytic efficiency, mode of action and basic chemistry have accelerated the possibility of using ribozyme human gene therapy to target specific human diseases. With the development of delivery systems well under way, and with much deeper understanding of molecular genetics of human disease, it is hoped that ribozymes will soon emerge as gene-targeted molecular therapy

Conclusion

Nucleic acid-based therapeutic drugs represent a promising and novel class of antiviral agents currently in development They offer significant therapeutic advantages over conventional antiviral drugs in that they are versatile, specific and efficacious. This review highlights a wide range of applications in which oligonucleotides are employed in antiviral therapy Antisense oligonucleotides, DNAzymes and ribozymes represent three main classes of antiviral oligonucleotides that are promising and attractive candidates for future clinical development. However, in order for these agents to fulfill their potential as medicines of the new millennium, a number of significant issues must be addressed. The biological stability of the first generation PS oligonucleotides, the safety of second and third generations of chemically modified oligonucleotides, and their general delivery and transport into intracellular sites of infection are focus of intense research and development efforts. These efforts have been greatly aided by advances in synthetic medicinal chemistry that results in the better design of oligonucleotides with improved stability and fewer side effects. These are currently tested in clinical trials. Formulation improvements have also been made to enhance intracellular delivery of oligonucleotides to sites where viruses replicate. Of particular interests is the use of novel liposome formulations that can protect oligonucleotides from nuclease degradation and target them intracellularly. Cationic and pH-sensitive liposomes are the formulations of choice for oligonucleotides. Overcoming these challenges mentioned above will enable nucleic acid-based drugs to reach their full potential as antiviral therapeutics.

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